Supplementary information

Tick saliva protein Evasin-3 allows for visualization of inflammation in arteries through interactions with CXC-type chemokines deposited on activated endothelium

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Experimental section

Materials and Methods

All chemicals were purchased from commercial sources and used without further purification: N^{α}-*tert*-butyloxycarbonyl (Boc) amino acids, Bachem (Bubendorf, Switzerland); 2-(6-Chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), Peptides International (Louisville, KY, USA); Methylbenzhydrylamine (MBHA)-polystyrene resin, ChemPep (Wellinton, FL, USA); Boc-Leu-PAM resin, Bachem (Bubendorf, Switzerland) or Applied Biosystems (Foster City, CA, USA); *N*,*N*-Diisopropylethylamine (DiPEA), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), diethylether, trifluoroacetic acid (TFA), and acetonitrile (CH_3CN), Biosolve (Valkenswaard, The Netherlands); methanol, *O*-methylhydroxylamine hydrochloride ($MeONH_2 \cdot HCl$), hydrogen fluoride (HF), thiophenol, benzylmercaptan, α-cyano-4-hydroxycinnamic acid, triisopropylsilane (TIS), tris(hydroxymethyl)aminomethane (TRIS; Trizma® base), guanidinium hydrochloride (Gdn HCl), Benzonase® Nuclease, and p-cresol, Sigma-Aldrich (St. Louis, MO, USA); Oregon Green® 488 Carboxylic Acid, succinimidyl ester, 6-isomer, Molecular Probes (Eugene, OR, USA); cystine, cysteine, and Bugbuster Merck KGaA (Darmstadt, Germany); maleimide-PEG11-Biotin, Thermo Fischer; Fondaparinux, GSK under commercial name Arixtra®.

Peptide fragment synthesis

All peptide fragments were synthesized by manual solid-phase peptide synthesis on a 0.1–0.25 mmol scale using the *in situ* neutralization/activation procedure for Boc-/Bzl-peptide synthesis as described previously [1,2]. However, instead of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), HCTU was used.

CXCL1 (Uniprot ID: p09341 35-107)

Human CXCL1, a 73 amino acid chemokine was synthesized as described before [3]. The modification in the C-terminal CXCL1 fragment was made to allow introduction of biotin-label. Here, the C-terminus consisted of H-[Cys^{35}-Asn^{73}-$Lys(Fmoc)^{74}$]-NH_2. After cleavage of the Fmoc-group using 20% (v/v) piperidine in DMF, an encrypted cysteine residue was coupled as a thiaproline residue on the resin to the ε-amino group of lysine.

After chain assembly, the peptides were deprotected and cleaved from the resin by treatment with anhydrous hydrogen fluoride HF for 1 h at 0 °C, using 4% (v/v) *p*-cresol as a scavenger.

Following cleavage, the peptides were precipitated with ice-cold diethylether, dissolved in a H_2O/CH_3CN/TFA mixture and lyophilized.

N56K Evasin-3 (Uniprot ID: p0c8e8 21-86)

The N-terminus H-Leu^{1}-Gly^{36}-3-mercaptopropionic acid (MPA) was synthesized on Boc-Leu-PAM (4-Hydroxymethyl-phenylacetamidomethyl) resin. MPA was coupled 2 times for 30 minutes. Deprotection of the trityl-group of MPA was done by treating the resin with a mixture of 95/2.5/2.5 TFA/TIS/H_2O.

The C-terminal H-[C^{37}-R^{66}]-NH_2 segment was synthesized on MBHA resin. After chain assembly, approximately 120 mg (0.067 mmol) of peptidyl-resin was treated with 20% (v/v) piperidine in DMF (3 x 4 min) for N^{ε}-Fmoc group removal. Subsequently, 5 mg (9.82 µmol) Oregon Green 488 carboxylic acid succinimidyl ester, 6-isomer (OG488) was dissolved in DMF and added to the Fmoc-deprotected resin. The reaction was performed overnight at 37 °C. Subsequently, the peptidyl-resin was washed with DMF and treated with TFA (2 x 5 s, 2 x 1 min) to remove the remaining N^{α}-Boc protecting group before the peptide was cleaved from the resin with HF as described above.

Native chemical ligation

Native chemical ligation of unprotected synthetic peptide segments was performed as follows: 0.1 M TRIS buffer, pH 8, containing 6 M Gdn-HCl was added to dry peptides yielding approx. 10 mg/mL of peptide fragments. Subsequently, 1% (v/v) benzylmercaptan and thiophenol were added. The ligation reaction was performed in a heating block at 37 ºC and the mixture was vortexed periodically to equilibrate the thiol additives. Reaction progress was analyzed on analytical HPLC and ESI/MS or with UPLC-MS. Ligated material was purified by HPLC, lyophilized and subjected to oxidative folding. Oxidative folding of the proteins was performed in 0.1 M TRIS buffer, pH 8.0, containing 1 M Gdn-HCl, and 1 mM cystine/8 mM cysteine as a redox couple. The reaction mixture was stirred at 4 °C, and after reaction completion, as detected with analytical HPLC, purified using a (semi)preparative HPLC system comprised of a Waters Deltaprep System consisting of a Waters Prep LC Controller and a Waters 2487 Dual wavelength Absorbance Detector (λ = 214 nm).

CXCL1 biotinylation

Folded CXCL1 containing a C-terminal thioproline residue was dissolved in 6 M Gdn-HCl, 0.1 M sodium acetate, 5 mM EDTA, pH 4.5 at the final concentration of 3.2 mg/ml and

deprotected with 40 mM methoxyamine. Simultaneously 10 equivalents of maleimide-activated PEG-11-biotin was added to the solution. The mixture was incubated at 37℃ for 4 hours. After reaction completion, CXCL1-biotin was purified and lyophilized.

Expression of hCXCL1 and Evasin-3

Evasin-3 and met-Evasin-3 (the variant contained N-terminal methionine M0) was expressed as described previously [4]. *E. coli* codon-optimized cDNA for human CXCL1 (UniProt: p09341, 35-107) in pET23a vector was obtained from GenScript (Piscataway, NJ, USA) and transfected to TSS chemically competent BL21 (DE3) pLysS cells. Single colonies were grown overnight in 5 mL of LB medium at 37 °C in the presence of 100 µg/mL of ampicillin as a starting culture and then transferred to 1 L of fresh LB media. Once the OD_{600} value reached 0.6-0.8, expression of target protein was induced with 0.1 mM or 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich). Cells were harvested 3 h after induction by centrifugation at 4000 rpm for 20 min at 4°C.

In order to obtain [^{13}C, ^{15}N] CXCL1, cells were grown in LB medium until OD_{600} reached 0.6-0.8. Cells were subsequently harvested by centrifugation at 4000 rpm at 4 °C and transferred to M9 medium. Prior to induction, cells were incubated in media for 1 h to deplete internal carbon and nitrogen sources. At induction, media was supplemented with 1 g/L of *U*-C6 [^{13}C]-glucose and $^{15}NH_4Cl$. ^{13}C-glucose concentrations in media samples were monitored using a generic blood glucose meter and cells were harvested by centrifugation after 80% of ^{13}C-glucose was consumed.

Subsequently, pellets were resuspended in 50 mM TRIS, pH 8 and lysed with 1x Bugbuster (Merck) and 0.1 U/ml Benzonase® Nuclease (Sigma-Aldrich). Cell debris was removed by centrifugation at 10000 rpm for 20 min at 4 °C, and the soluble fraction was dialyzed overnight against 0.5% (v/v) acetic acid using 3.5 kDa membrane. Precipitates were removed by centrifugation at 10,000 rpm for 20 min at 4 °C and the supernatant was lyophilized.

Lyophilized material was dissolved at 20 mg/mL in 0.1 M TRIS, pH 8, containing 6 M Gdn-HCl and added dropwise to 0.1 M TRIS buffer, pH 8, containing 1 M Gdn-HCl, 10 mM cysteine, and 1 mM cystine to a final concentration of 1 mg/mL and stirred overnight at 4 °C. After completion of folding, the protein was purified by preparative HPLC, analyzed by Waters UHPLC XEVO-G2QTOF system, and lyophilized.

NMR sample preparation

NMR samples of uniformly labeled [^{15}N, ^{13}C] CXCL1 were measured at different concentrations: 600, 250 and 50 µM, all three samples prepared in 3 mm NMR tubes (160 µl volume). Concentrations of CXCL1 were based on stock solutions made from dry weight lyophilized CXCL1, because accurate UV-based concentrations were not possible due to the absence of aromatic residues in CXCL1. NMR samples contain 25 mM sodium acetate-d^3 buffer (pH 4.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM sodium azide, 6 µM 4,4-dimethyl-4-silapentane-1-sulfonic acid-d^6 (DSS-d^6) as chemical shift reference, and 2% (v/v) D_2O for deuterium lock.

Complexes between [^{15}N, ^{13}C] CXCL1 and met-Evasin-3 were prepared by addition of concentrated met-Evasin-3 stock solution made in the same buffer as the hCXCL1 NMR samples. Final concentrations of the titrated complexes were approximately 20% lower than for the free CXCL1 samples, and were ~200 µM and ~40 µM. Correct stoichiometry of the [^{15}N, ^{13}C] CXCL1/met-Evasin-3 complexes in the slow exchange regime were checked by 1D proton- and heteronuclear NMR to ensure that there is always a slight excess of free met-Evasin-3 proton resonances present in the spectrum of the complex, with no remaining signals of [^{15}N, ^{13}C] CXCL1 visible. Fondaparinux-binding experiments were carried out by addition of concentrated GAG stock solution to the [^{15}N, ^{13}C] CXCL1 and [^{15}N, ^{13}C] CXCL1/met-Evasin-3 complex.

NMR spectroscopy

NMR spectra of hCXCL1 and its met-Evasin-3 complexes were recorded on a Bruker Avance III HD 700 MHz spectrometer, equipped with a TCI [^{13}C,^{15}N,^{1}H] cryoprobe. Probe temperature was set to 37 °C, internally calibrated by a small thermocouple placed inside a 5 mm NMR tube filled with water and inserted into the probe.

For a 0.6 mM hCXCL1 sample, a 1D proton spectrum, 2D ^{15}N-^{1}H HSQC, ^{13}C-^{1}H constant-time HSQC, 2D C_CON, and a series of 3D triple resonance spectra (HNCO, HNcaCO, HNCACB, CBCAcoNH, HBHAcoNH, 2D C_CON) were recorded to assign most of all ^{1}H, ^{13}C and ^{15}N backbone resonances. Water suppression was carried out using excitation sculpting [5], while gradient sensitivity-enhanced versions of ^{13}C-^{1}H HSQC, ^{15}N-^{1}H HSQC (flip-back version) and gradient triple resonance experiments from the standard Bruker pulse sequence library were used throughout. Bruker type 3D HCCh-DIPSI, 3D-^{15}N-edited- and 3D-^{13}C-edited NOESY spectra were further used to assign virtually all remaining side chain resonances. The mixing time for these three 3D spectra was set to 13 ms, 150 ms and 150 ms, respectively.

Stereo-specific assignments of prochiral methyl and methylene groups were not analyzed *per se* and ^{1}H chemical shifts of prochiral pairs are reported in arbitrary order going from high to low ppm value. At lower protein concentration in combination with solution conditions of pH 4.5 and 37 °C, *apo* hCXCL1 displayed well-separated amide peaks that correspond to the dimeric and monomeric form of the protein that are simultaneously present in slow exchange. Assignment of those minor monomer peaks in the ^{15}N-^{1}H HSQC spectrum recorded at 0.6 mM could be established from symmetric exchange peaks to already assigned dimeric amide proton resonance positions in the 3D ^{15}N-edited NOESY. At 40 µM protein concentration the equilibrium between monomer:dimer reached an almost 1:1 ratio and an additional 3D-^{15}N-edited NOESY (100 ms mixing time) recorded on this low concentration sample was used to better resolve monomer peaks now having less overlap with respect to dimer peaks.

The same set of NMR spectra was recorded for the complex of 200 µM [^{15}N, ^{13}C] CXCL1/met-Evasin-3 in order to assign all CXCL1 resonances in the complex. In the complex, only one distinct set of signals of labeled CXCL1 is observed at both protein concentrations of 200 µM and 40 µM, and spectra turned out to be identical independent of protein concentration.

Spectral processing was done by Topspin 3.2 (Bruker GmbH, Rheinstetten, Germany) spectral analysis and resonance assignment was carried out by Sparky 3.115 [6]. Zero-filling and forward linear prediction was routinely used before each Fourier transformation to get increased resolution in the indirectly detected dimension.

Analysis of chemical perturbations in terms of 3D structure were visualized on the solution structure of the full-length human CXCL1 protein dimer (PDB code: 1MGS) [7].

Surface Plasmon Resonance

Direct binding analysis was performed on a Biacore T200 SPR apparatus (GE healthcare, USA). Therefore, CXCL1-PEG-biotin was immobilized to a SAHC 200M chip (XanTec bioanalytics GmbH, Düsseldorf, Germany) as instructed by the manufacturer's protocol to a final immobilization level of 60 RU. Flow cell 1 was left untreated and was used as a reference cell. For binding analysis, measurements were conducted at 30 µl/min flowrate, 250 s contact time, and 300 s dissociation time in PBS buffer pH 7.4 supplemented with 0.05% (v/v) Tween 20. Varying concentrations of compound, as indicated in the results section, were injected. For regeneration a 2M NaCl solution was used. Prior to every run, the apparatus was primed five times. Sensorgrams were analyzed with the Bia-evaluation software (version 1.1.1, Biacore company AB, Sweden) using a steady-state affinity equation with linear component for the interaction between Evasin-3 derivatives and CXLC1.

Evasin-3 adhesion assay

Human microvascular endothelial cells (HMVECs) were cultured in µ-slides I 0.4 (Ibidi GmbH, Martinsried, Germany). After 24 hours of static culturing, the slides were installed in the Ibidi pump system with the fluidic units at 37 °C. HMVECs in experimental conditions were subjected to 2.0 Pa shear stress for 72 hours, whereas in control conditions HMVECs were cultured statically for the same period of time. Subsequently, the cells were incubated with 20 ng/mL OG488-labeled Evasin-3 for 20 minutes and Hoechst nuclear staining for 5 minutes at 37 °C. After thorough washing, fluorescent images were taken with the Pathway 855 bioimaging system (Becton Dickinson, Franklin Lakes, NJ, USA) and results were analyzed with AttoVision software (Becton Dickinson).

Two-photon microscopy

For TPLSM imaging, segments of common carotid arteries of a surplus mouse (length ~6–8 mm) were freed of adipose and connective tissue and mounted on two glass micropipettes in a homebuilt perfusion chamber (IDEE, Maastricht, The Netherlands) as described previously[8,9]. Carotid arteries were perfused with 10 µM LPA 20:4 (Avanti Polar Lipids, Alabaster, AL, USA) in 3-(*N*-morpholino)propanesulfonic acid (MOPS; Acros Organics, Geel, Belgium) buffer pH 7.4 or MOPS buffer alone at 0.540 mL/min for 10 min. Subsequently, arteries were successively perfused with N56K Evasin-3 OG 488 (5 ug/mL; 0.7 µM) and MOPS buffer under the same conditions. N56K Evasin-3 OG 488 deposition was imaged with a Bio-Rad 2100MP (Hemel Hampstead, UK) TPLSM. The excitation source was a Spectra Physics Tsunami Ti:Sapphire laser (Mountain View, USA), tuned and mode-locked at 800 nm. Laser light reached the sample through the Nikon water immersion lenses (60X, numerical aperture (NA) 1.0, working distance or 60X, NA 1.2), incorporated in an upright Nikon E600FN microscope (Tokyo, Japan). Further magnification was achieved by optical zoom. Two photomultipliers detected the fluorescence. Each photomultiplier accepted a different, tunable wavelength region, which were color-coded: blue (Second harmonics generation of collagen, 400-450 nm); green (for Evasin OG488, 500-560 nm). TPLSM data were analyzed with ImageJ 1.51k software package[10].

PMN isolation and migration

Human neutrophils were isolated from the blood of healthy donors as described previously [11]. All participants provided informed consent according to the Helsinki Declaration. Isolated neutrophils were resuspended at ($1x10^6$/mL) in RPMI 1640 medium supplemented with 1%

(v/v) fetal calf serum. A 12-well chemotaxis chamber with a 5 µm polycarbonate membrane (Neuro Probe Inc., Gaithersburg, MD, USA) was used to assess the migration of neutrophils towards the chemoattractant CXCL1 (1 nM) in presence or absence of Evasin-3 (10 nM). The chemoattractant was added in the lower wells and the neutrophils ($1x10^5$ cells) were seeded in the upper wells. After 90 min incubation in a humidified atmosphere (5 % CO_2/95 % air, 37 °C, the non-migrated cells were carefully removed and the membrane was stained with Diff-Quick stain (Eberhard Lehmann GmbH, Berlin, Germany). Migrated cells were visualized by light microscopy, counted manually in five fields of view and expressed as cells/mm^2.

THP-1 monocytic cells

Human acute monocytic leukemia cell line (THP-1) monocytic cells were purchased from Leibniz-Institute DSMZ (ACC 16). Cells were cultured in RPMI glutamax medium (Gibco Thermo Fisher) supplemented with 20 % fetal calf serum (Gibco Thermo Fisher), 1 % penicillin/streptomycin (Gibco Thermo Fisher). Cells were cultured in a humidified atmosphere (5 % CO_2/95 % air, 37 °C) and routinely passed by transferring a certain amount of cell suspension to fresh medium at a concentration of 1:10 (v/v).

Flow chamber adhesion assay

For flow chamber adhesion, human umbilical vein endothelial cells (HUVEC) were seeded in 35-mm, pre-coated collagen (30 µg/mL) culture dishes and activated with LPA (10 µM) for 4 h. Human neutrophils, isolated as described above, and monocytes were labeled with 1 µM green fluorescent nucleic acid stain (Syto 13) for 30 min at 37 °C, washed and perfused in Hank's buffer, containing 10 mM Hepes, pH 7.45, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.2% human albumin for 2–6 min at 3 dynes/cm^2. To evaluate the effect of Evasin-3 on neutrophil adhesion, Evasin-3 (5 µg/mL; 70 nM) was perfused for 6 min at 3 dynes/cm^2. Adherent fluorescent cells were manually counted in >6 fields of view and expressed as cells/mm^2.

Chemokine array of HUVEC cells

For chemokine expression HUVEC cells ($1x10^6$ cells/per dish) were seeded in 100-mm, pre-coated collagen (30ug/ml) culture dishes and incubated with LPA (10 µM) for 4 h at 37 °C, and 5 % CO_2/95 % humidity. After incubation, medium was collected and centrifuged for 5 min at 95000g. The chemokine array was performed according to the manufacturer's instructions. Human Chemokine Antibody array (ab169812), Abcam (Cambridge, United Kingdom)

Statistical Analysis

All mean values are given as ± SD (standard deviation). Statistical analysis was performed with GraphPad Prism software (version 7.02; GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) or repeated measures ANOVA was used to compare groups. The level of significance was set at p less than 0.05.

Supplementary results

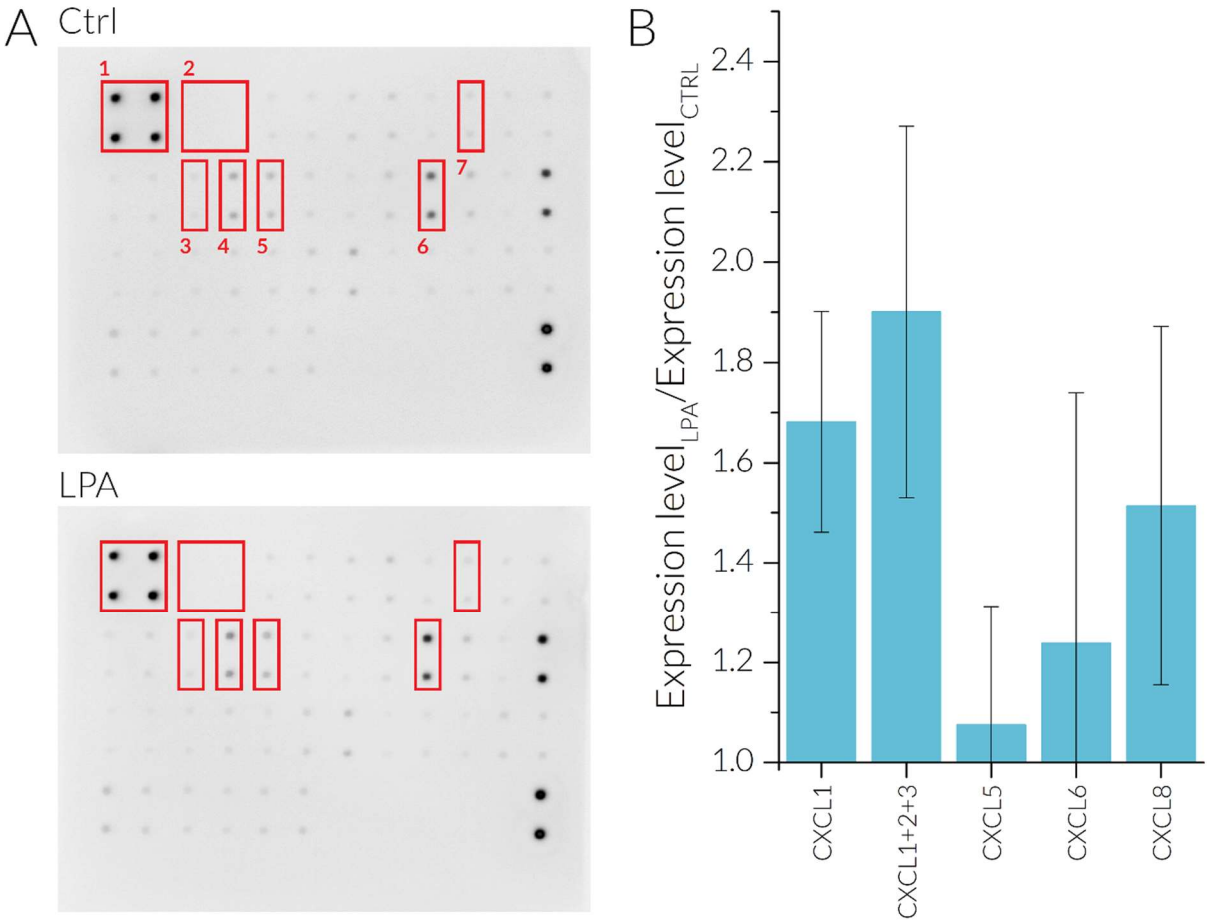

Figure S1. Expression of chemokines by ECs before and after activation by LPA. A. Chemokine array membranes before (top) and after (bottom) LPA activation. By red rectangles are highlighted: 1 – positive controls; 2 – negative controls; 3 – CXCL6; 4 – CXCL1, CXCL2, CXCL3; 5 – CXCL1; 6 – CXCL8; 7 – CXCL5. **B.** Relative expression level of CXC-type chemokines after LPA activation based on 2 control and 2 activated membranes (4 spots in total for each chemokine).

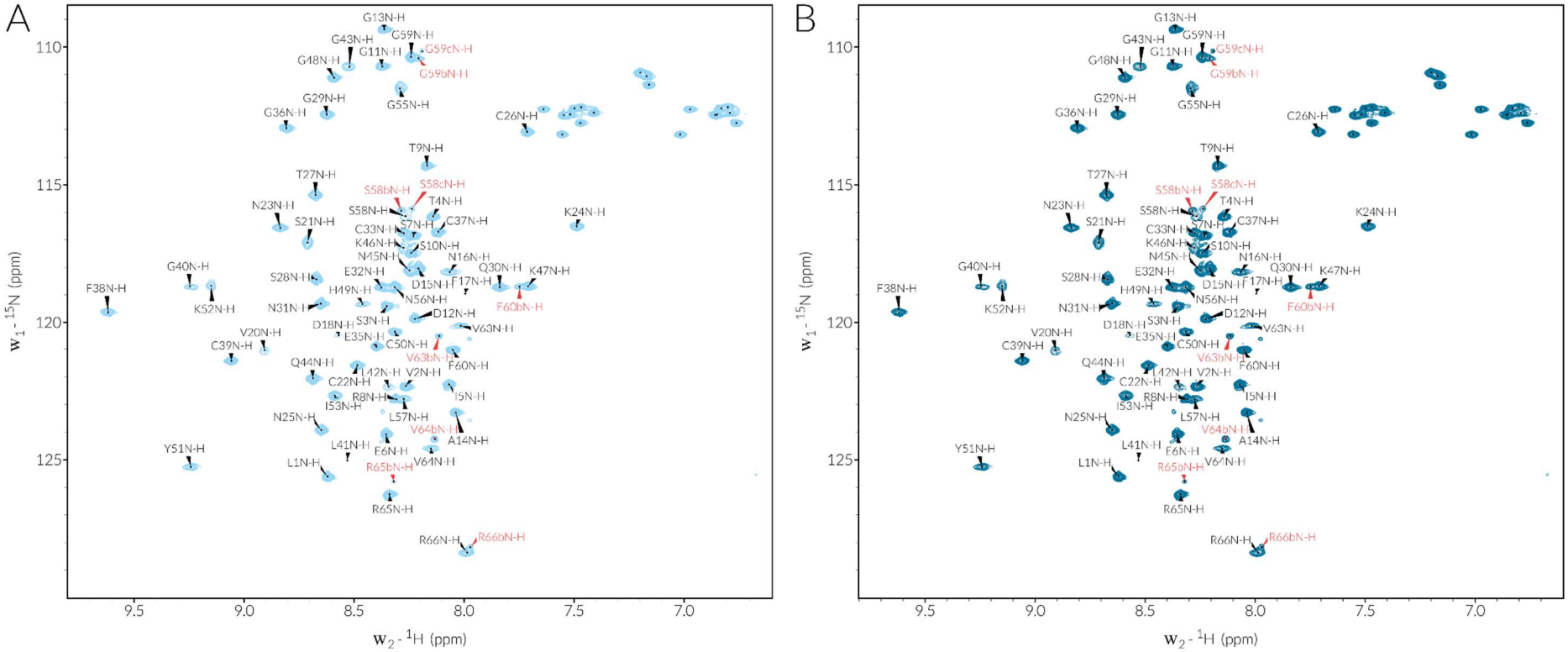

Figure S2. Binding of CXCL1 to Evasin-3 complex. ^{15}N-^{1}H HSQC spectra of 50 µM [^{15}N, ^{13}C] met-Evasin-3 (A) and 350 µM CXCL1/[^{15}N, ^{13}C] met-Evasin-3 complex at 37°C, pH 4.5. Assignments of asparagine, glutamine, and arginine side chain peaks are left out for improved visibility.

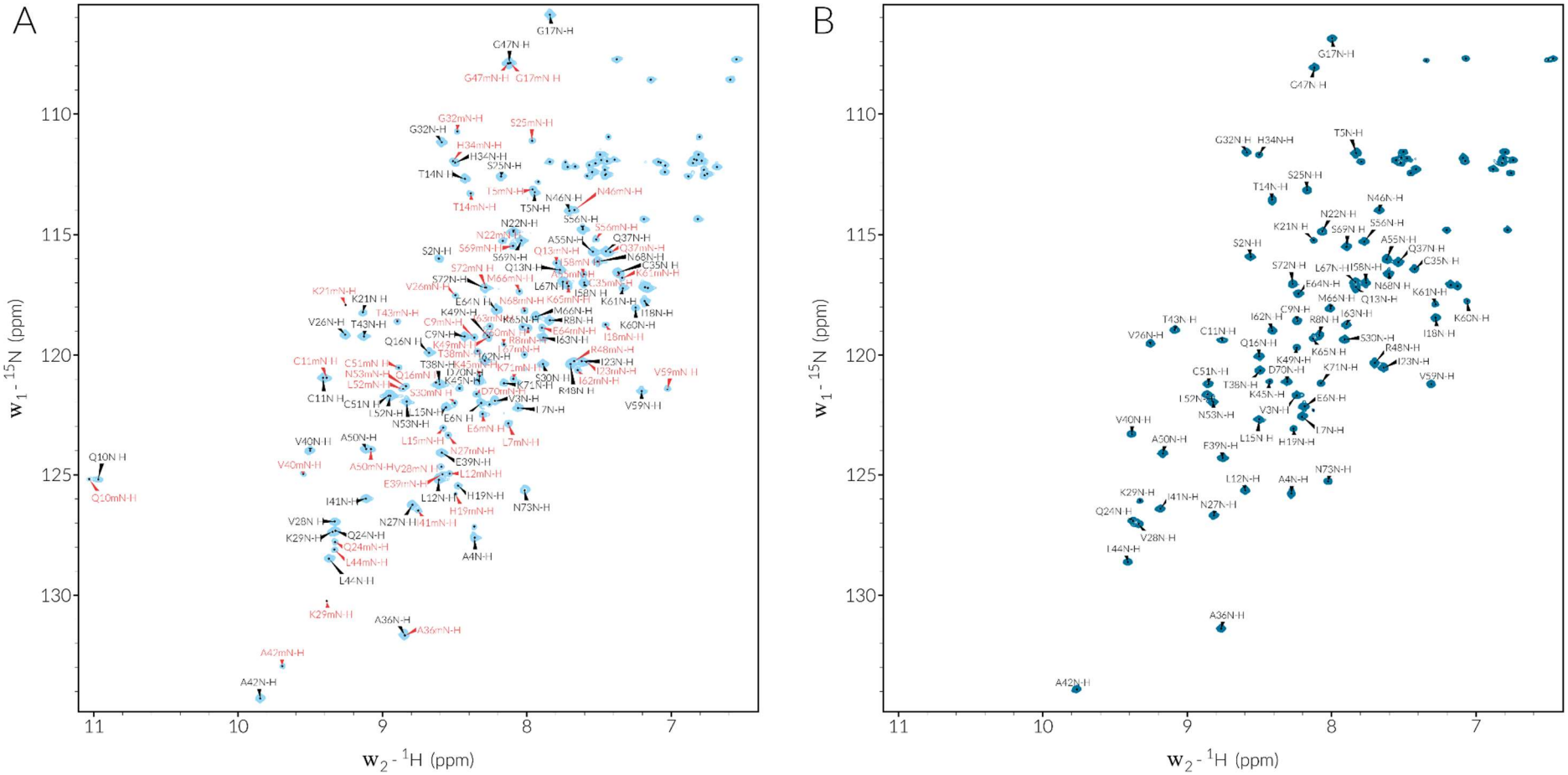

Figure S3. Binding of Fondaparinux to CXCL1. ^{15}N-^{1}H HSQC spectra of 50 µM [^{15}N, ^{13}C] CXCL1before (A) and after (B) addition of 125 µM of Fondaparinux at 37°C, pH 4.5. Amide peaks of the monomeric form are labelled in red; the dimeric form – black. Assignments of asparagine, glutamine, and arginine side chain peaks are left out for improved visibility.

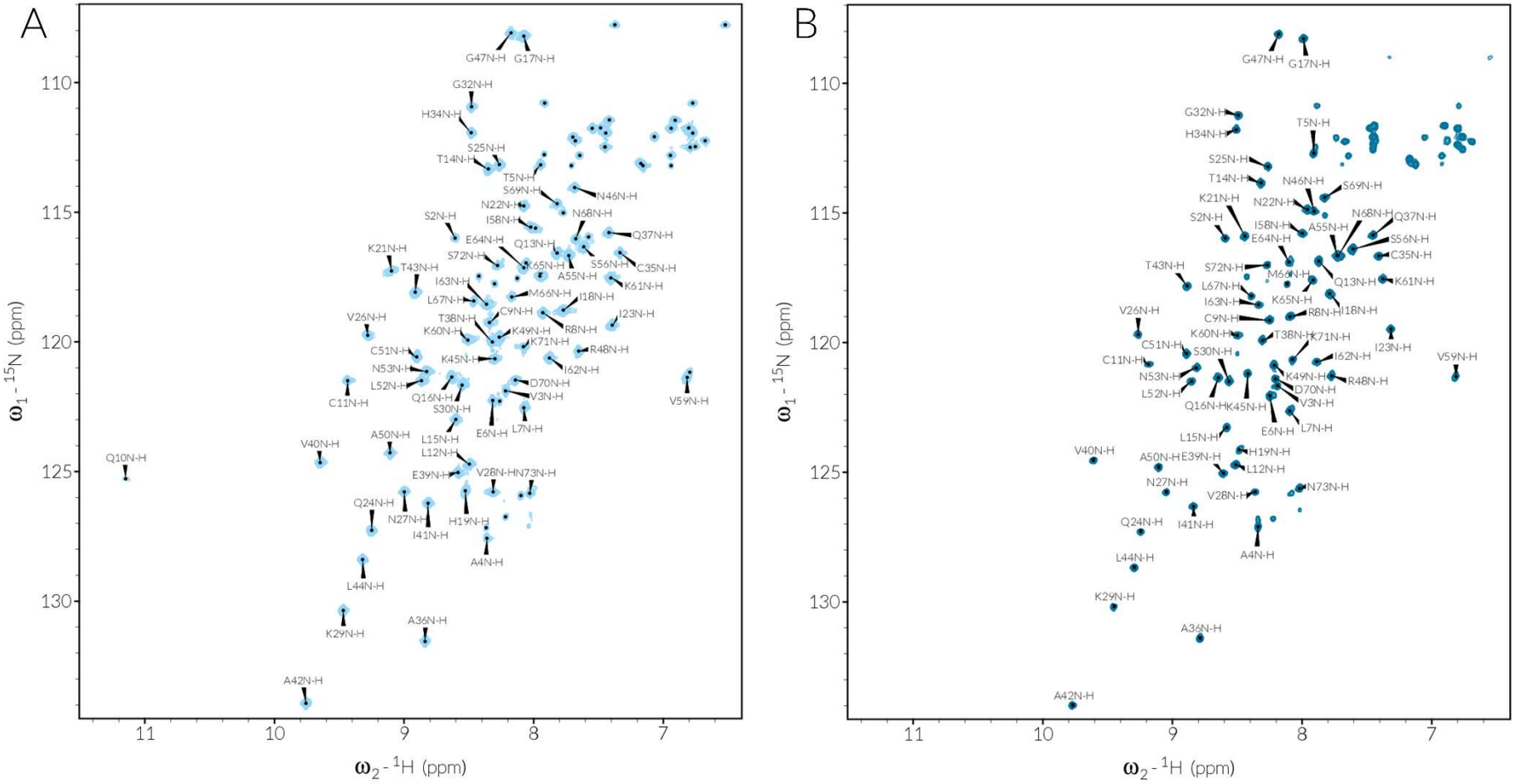

Figure S4. Binding of Fondaparinux to CXCL1/Evasin-3 complex. ^{15}N-^{1}H HSQC spectra of 250 µM [^{15}N, ^{13}C] CXCL1/met-Evasin-3 complex before (A) and after (B) addition of 1.25 mM of Fondaparinux at 37°C, pH 4.5. Assignments of asparagine, glutamine, and arginine side chain peaks are left out for improved visibility.

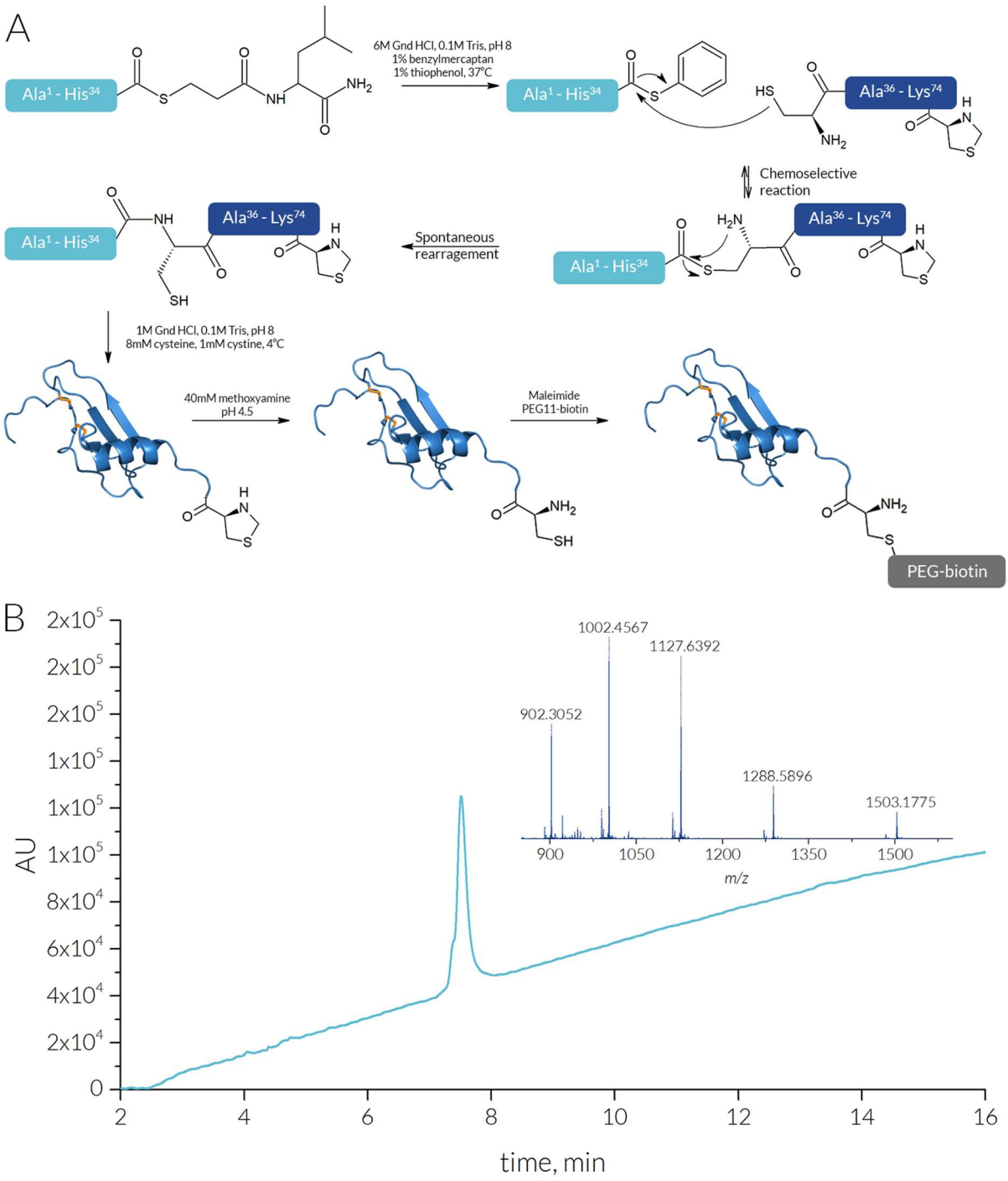

Figure S5. Synthesis overview of CXCL1-PEG-biotin for SPR biosensor analysis. A. The scheme of CXCL1-PEG-biotin synthesis. **B.** LC-MS profile of purified synthetic CXCL1-PEG-biotin. Deconvoluted and calculated mass of $[M+H]^+$ were 9007.71 Da and 9009.43, respectively.

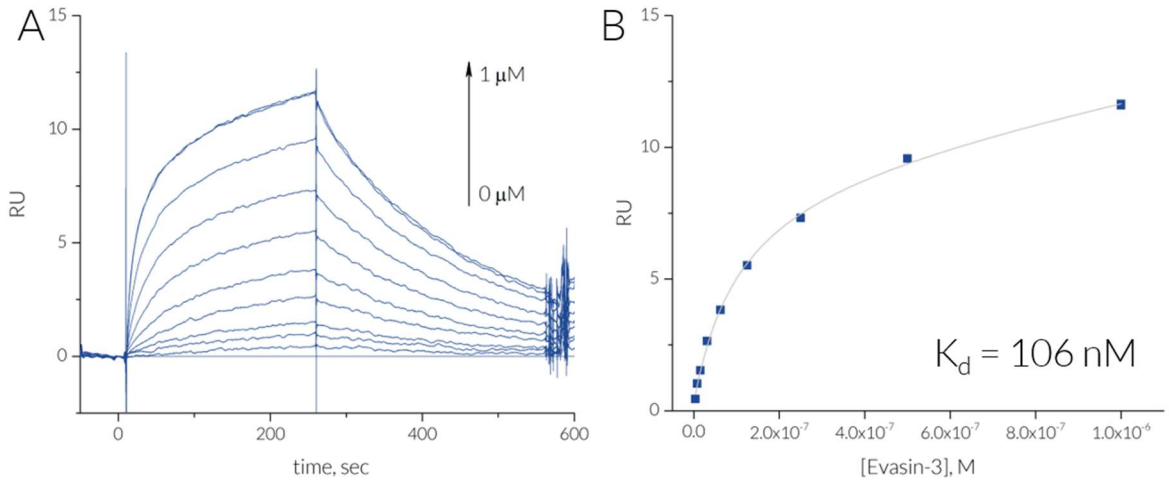

Figure S6. Affinity of Evasin-3 to CXCL1. Sensorgrams (A) and fitting curves (B) of Evasin-3 (**C**) with immobilized CXCL1 on a streptavidin-coated chip. Apparent K_D value was calculated to be 106 nM, R_{max} - 9.1, Chi^2 - 0.025.

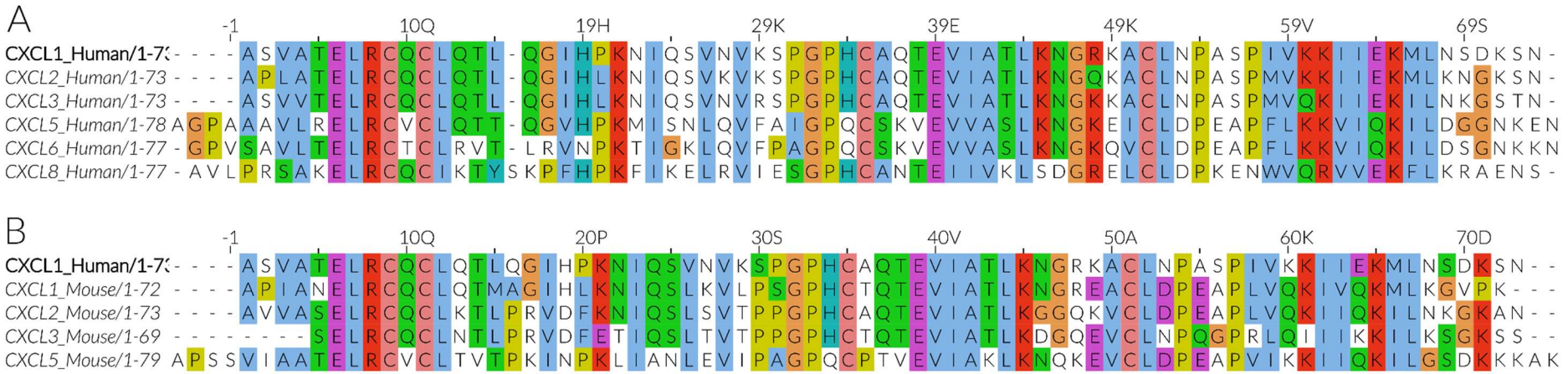

Figure S7. Sequence alignment of human CXC-type chemokines (A) and human CXCL1 and murine CXC-type chemokines (B).

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